COBALT 60 RADIATION AND NITROGEN UTILIZATION BY THE CLADONIA CRISTATELLA PHYCOBIONT TREBOUXIA ERICI

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Summary

Trebouxia erici Ahmadjian, the phycobiont of the lichen Cladonia cristatella Tuck., was grown in nitrogen modified media and irradiated by exposure to Co^{60} in doses of 4, 40, and 480 kr. The alga was spectrophotometrically analyzed for changes in chlorophyll and phaeophytin content 4 and 8 d after irradiation. Dry weights of the cultures were also measured. After irradiation, chlorophyll content decreased over the 5 d period in all cases. The magnitude of the decrease in chlorophyll 'a' was inversely related to the radiation dose. The effect of radiation of the chlorophyll content was minimal and few changes were noted on the growth rate as measured by the dry cell weight. However, the 480 kr dose reduced the actual chlorophyll 'a' content of the cultures, while change in the quantity of chlorophyll 'b' was less affected. $(NH_4)_2SO_4$, KNO_3, l-alanine, and NH_4NO_3 had greater effects on the phycobiont compared with Co^{60} induced changes. The pigments in the cultures were chlorophylls 4 days after irradiation, and mostly phaeophytins at 8 days. Cobalt 60 was not a significant factor in degrading chlorophyll to phaeophytin, apparently change was related to the growth phase of the culture. Certain nitrogen sources such as KNO_3 produced little effect in the change of chlorophyll to phaeophytin while with other sources such as l-alanine, complete conversion occurred.

Introduction

Lichens are known to be radioresistant. Hale (1967) found some species of lichens able to tolerate 10 to 12 kr/d for 3 yrs, while 11 species survived 2,250 r/d for 32 months (Woodwell and Whitaker, 1968). Various explanations identify lichen radioresistance. Hawksworth and Rose (1976) suggested that frequent dry dormant periods to which lichens are prone help increase their tolerance to radiation. Gannutz (1968) found both lichen symbionts and thalli were more radiosensitive when dry, while Brode (1964) attributed the radioresistance of lichens to diffuse meristematic tissue and the small nuclei of the fungal symbiont.

Few studies of radiation effects on lichen phycobionts have been made. Gannutz (1968) found reduced growth in Lecanora lathimii, Lesides varians, and Candelaria concolor, but not their isolated symbionts. He proposed that the radiosensitivity of the phycobiont

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was the limiting factor in the survival of irradiated lichens, however, the lichen thallus was more radiosensitive than either symbiont. Gannutz (1968) found a marked reduction in the DNA content of T. erici irradiated with 100 kr of gamma radiation.

The effects of ionizing radiation on free living algae are well known (Godward, 1962). In general, algae are more radioresistant than higher plants Some cells may survive exposure to 2 million rads and continue to grow indefinitely. Zill and Tolbert (1958) found decreases in CO_2 fixation and O_2 evolution during photosynthesis in irradiated Chlorella phyenoidosa.

Certain chemicals protect or aid recovery of irradiated algae. Thiourea, cysteine, and anaerobiosis produced by bubbling nitrogen through culture media protected algae from X-irradiation (Godward, 1962). Lawrence (1971) indicated nutrient treatments after irradiation effected cellular radiation damage in algae.

Modification of the nitrogen source effected the growth of T. erici and possibly effected the algal response to irradiation in the visible range, thus Fox (1967) reported increased growth on organic nitrogen compounds and reduced nitrogen sources for 3 species of Trebouxia. Ahmadjian (1967) also found better growth of Trebouxia on organic nitrogen sources, but only when a carbon source was included. Fox noted the best growth of T. erici on media containing arginine, followed by alanine, casamino acids, proline, and guanosine.

The current study was designed to examine the effects of nitrogen sources on the response of T. erici to irradiation. Changes in the dry weight and chlorophyll content of colonies were studied.

Materials and Methods

Algal cultures and growth conditions

Axenic cultures of the lichen phycobiont T. erici were obtained from the collection of Dr. Vernon Ahmadjian, Clark University, Worcester, Massachusetts. Algal cells were grown in 25 x 150 mm screw cap glass culture tubes containing 28 ml of modified Bold's Basal Medium (Ahmadjian, 1967). Culture tubes were divided into 8 sets of 14 tubes. One tube in each set contained no nitrogen source while the other 13 each contained one of the following nitrogen sources, equivalent in N content to 0.75 g/l $NaNO_3$: l-alanine, l-arginine, l-asparagine, casein, KNO_3, l-lysine, $NaNO_3$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, peptone, tryptophan, urea. All media were sterilized in an autoclave at 121 C for 20 min except that which contained urea. The urea solution was sterilized in a Seitz filter and added to autoclaved media.

Tubes containing the media were inoculated with suspensions of cells grown in liquid medium with $NaNO_3$ as the nitrogen source. Each set of tubes was placed in separate baskets and illuminated from below with fluorescent light providing 550 lux of constant illumination. Cultures were incubated at 20 C.

Two sets of culture tubes each were irradiated at 4, 40, and 480 kr acute radiation at the Phoenix Memorial Laboratory, The Univ. of Michigan. A radiation source of Co^{60} was used. Irradiation was carried out 24 d after inoculation during log phase of growth. Two sets of tubes were not irradiated and served as controls.

Dry weight and chlorophyll content analysis

On day 4 and 8 after irradiation the cultures were analyzed for chlorophyll content using a spectrophotometric analysis adapted from Strickland and Parsons (1972). Four drops of a solution of Na_2CO_2 at pH 11 were added to each tube to prevent the degradation of chlorophyll to phaeophytin. Algal cells were then centrifuged using a Vortex Jr. mixer. The volume of the sample was measured and divided in two. One half of each cell culture was filtered using a tared millipore HA filter (pore size, 45 um), dried to a constant weight at 60 C, and weighed, while the other half was filtered on an unweighed millipore filter.

Each of the unweighed filters with the collected cells was ground with approximately 6 ml of 90% acetone in a glass tissue grinder. It was further ground in a small mortar with a pestle in the presence of a small amount of quartz sand and 90% acetone. The 90% acetone was prepared by shaking reagent grade acetone (B.P. 56.6 C with granular, anhydrous sodium carbonate, decanting the acetone into a dark bottle and mixing in a ratio of 9:1, acetone:distilled water. The solution was transferred to a 15 ml screw cap centrifuge tube and stored at 4 C in the dark for 40 h to extract the chlorophyll. After extraction the sample was allowed to come to room temperature in the dark and was then centrifuged at 3,000 rpm for 10 min. The sample was then brought to 10 ml volume in a fresh tube by adding 90% acetone. A portion was then transferred to a 1.0 cm path length cuvette. The absorbance of the solution was measured against an acetone - filter blank at wave lengths of 750, 665, 645, and 630 nm through a slit width of 0.5 nm with a Varion Techtron spectrophotometer. To determine the quantity of phaeopigments, the solution was acidified by the addition of 2 drops of dilute HCl (concentrated HCl: distilled water, 1:1) per cuvette. The absorbance of the solution was then remeasured at 665 and 750 nm. A ratio of absorbance after acidification (a) to the absorbance before acidification (b) was made for each sample at the designated wavelengths by using the equation $665_b-750_b/665_a-750_a = \alpha$ Acidification of a solution of pure chlorophyll 'a' results in a 40% reduction in its optical density at 665 nm. Samples with α equal to 1.7 contained little phaeophytin while those of pure phaeophytin yield α equal to 1.0 (American Public Health Association, 1971). Contents of chlorophylls 'a' and 'b' were determined using the following equations: Chl. 'a' (mg/l extract) = $11.6\ A_{665} - 13.1\ A_{645} - 0.14\ A_{630}$; and Chl. 'b' (mg/l extract) = $20.7\ A_{645} - 4.33\ A_{665} - 4.42\ A_{630}$, where A = the absorbance of the extract at the wave length (nm) indicated by the subscript. The absorbance readings at 750 nm were used as a correction for turbidity by subtracting any value measured at that wavelength from the absorbance values at the

other wavelengths. Chlorophyll values for each sample were determined and expressed in terms of the dry weight of the sample by weighing the other half on a Mettler analytical balance. Samples were analyzed in replicates of three. Mean values were then used for a computer analysis of frequency distributions of values at all radiation levels and the control versus those recorded at 480 kr exposures only. Least squares multiple regression analysis was used to determine the strongest factors affecting chlorophyll content using a computer and SPSS program (Nie et al., 1975).

Results and Discussion

Dry weights

Table 1 shows the dry weight of T. erici cultures after 28 and 32 d growth. It can be seen that $(NH_4)_2SO_4$, KNO_3, l-arginine, and NH_4NO_3 promoted the most rapid growth. Rapid algal growth on media containing ammonium salts has been documented in previous studies but rapid growth on KNO_3 is somewhat unusual. Most green algae do not prefer nitrate containing media (Round, 1965). Results showed that exposure to radiation affected the rate of increase of the dry weight over time. Even if the algae were killed by the radiation, the weight of the cell materials would be about the same as a control culture of the same age. Cultures with a majority of dead or reproductively incapacitated cells can not increase, limiting their potential for weight increase over time.

As can be seen in Table 2, the percent change in dry weight of the algae from 4 to 8 d after irradiation produced little change at 4 and 40 kr, however a decrease was noted at 480 kr. Algae grown on some media, such as l-arginine and peptone, had reduced growth rates in the non-irradiated state (Table 1). There was not clear interaction between nitrogen source and radiation exposure with respect to changes in dry weight. Radiation may well have accelerated the onset of the senescent growth phase of the cultures, by killing or disabling cells.

Chlorophyll content

Radiation exposure, when compared to media type, was not a major factor in determining the chlorophyll content of T. erici at the specific dates of analysis (Tables 3,4). KNO_3, $(NH_4)_2SO_4$, NH_4NO_3, and NH_4Cl had important effects on the chlorophyll content of the algae. Exposure to 480 kr radiation reduced the actual amount of Chl. 'a', but its effects were much less pronounced than the effects of the nitrogen sources listed.

Table 5 shows that the reduction in Chl. 'a' content from 4 to 8 d after irradiation is inversely related to the radiation dosage, as were the changes in dry weight. The correlation was less apparent with Chl. 'b'. Mutated cells, with various changes in pigmentation, are known to occur in the progeny of irradiated algae (Zakharov and Tugerinov, 1964). Although some destruction of pigment in the T. erici cultures by radiation exposure may occur, the observed

effects appear related to changes in growth rate. Algae grown under the nonirradiated control condition showed the greatest decrease in Chl. 'a' content from 4 to 8 d after irradiation (Table 5). It seems unlikely that radiation was the major factor in changes of pigment quality. Increases in cell chlorophyll content lagged behind gains in dry weight. The control cultures, unaffected by radiation, continued to grow rapidly and had a loss of pigment for the interval between 30 and 34 d after innoculation. Reduced growth in the irradiated cultures having a stable chlorophyll content would account for a smaller differential between the weight of chlorophyll and cell weight as the radiation dose increased.

Table 5 shows the radiation effect is different for the two types of chlorophyll, therefore modification in dry weight gains by radiation can not completely explain the results. The least relative reduction in Chl. 'b' from day 4 to day 8 after irradiation was 40 k. Less degradation of Chl. 'b' is apparent. On the other hand, the production of the pigment may be stimulated. Effects on the RNA or enzyme systems could be responsible since the biosynthesis of Chl. 'b' involves a sequential oxidative step from Chl. 'a' (Meeks, 1974), and radiation could effect this, or another, step in the process.

Phaeophytin 'a' content

The non-photosynthetic phaeophytin pigment that absorbs light at the same wavelengths as Chl. 'a' can be estimated by converting all of the Chl. 'a' to phaeophytin 'a' by acidification. Solutions of pure Chl. 'a' show approximately 40% reduction in optical density when converted to phaeophytin 'a' (American Public Health Asso., 1971), and demonstrate no reduction in optical density when acidified. Table 6 presents the results of acidification of all chlorophyll solutions of the isolates as well as the means within radiation doses with all media. These results identify the amount of Chl. 'a' and phaeophytin 'a'. Percentage values close to or equalling 40% identify a large reduction in optical density indicating the presence of mostly Chl. 'a'. Percentage values near or equal to 0 showed no reduction in optical density of the solution by the addition of acid, therefore most of the pigment was phaeophytin 'a'. Some solutions showed a reduction in optical density slightly greater than 40% due to the sensitivity of the spectrophotometric method. All values above 40% were standardized to that value for purposes of analysis.

Mean values within radiation doses were all close to 32% 4 days after irradiation, indicating a greater proportion of Chl. 'a' than phaeophytin 'a' in the solutions (Table 6). At 8 days however, all reductions dropped to only about 9% indicating a natural degradation of Chl. 'a' to phaeophytin 'a' regardless of radiation dose. Examining the means for each medium, KNO_3 showed little degradation of Chl. 'a' from 4 to 8 days after irradiation. NH_4NO_3, $(NH_4)_2SO_4$, and l-lysine media produced algal cells with pigment as phaeophytin, initially showing optical density reductions after

acidification of 23%, 24%, and 13% respectively. Some conversion of Chl. 'a' to phaeophytin 'a' from day 4 to day 8 was noted with $NaNO_3$, while a greater change was noted with l-alanine which contained pure Chl. 'a' 4 days after irradiation and pure phaeophytin at 8 days.

Nitrogen sources had a more pronourced affect on the alga than did radiation exposure. Radiation appeared to modify growth. Affects on the chlorophyll content were relative to weight changes. The source of nitrogen in the medium did not protect T. erici against radiation effects, nor did it enhance those effects.

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Table 1. Mean dry weights of Trebouxia erici grown in 14 ml of nitrogen modified media

Medium	Mean dry weight (mg) A	B	Percent change
l-Alanine	4.98 (6.68)	6.77 (7.68)	+36 (+15)
No nitrogen	3.73 (3.39)	4.83 (4.91)	+29 (+45)
$NaNO_3$	7.87 (5.67)	9.93 (11.44)	+26 (+102)
NH_4NO_3	8.72 (10.63)	10.68 (12.73)	+22 (+20)
Tryptophan	4.50 (3.54)	5.30 (7.38)	+18 (+108)
NH_4Cl	7.64 (4.38)	8.94 (10.63)	+17 (+143)
l-Lysine	4.06 (3.60)	4.51 (3.66)	+11 (+2)
Urea	7.91 (9.35)	8.64 (8.52)	+9 (-9)
KNO_3	9.52 (8.55)	10.16 (11.78)	+7 (+38)
l-Asparagine	7.38 (7.23)	7.73 (7.88)	+5 (+9)
Peptone	6.67 (6.90)	6.78 (6.77)	+1 (-2)
l-Arginine	9.22 (10.20)	8.07 (5.71)	-12 (-44)
$(NH_4)_2SO_4$	10.49 (11.23)	9.11 (9.06)	-13 (-19)
Casein	5.75 (4.26)	4.49	-14

Means calculated from control and 3 radiation dose values. Control values in parentheses. A - 28 days after inoculation and 4 days after irradiation. B - 32 days after inoculation and 8 days after irradiation.

Table 2. Mean dry weights of Trebouxia erici colonies grown in 14 ml of all nitrogen modified media

Radiation dose (kr)	Mean dry weight (mg) Days after irradiation 4	8	Percent change
Control	6.83	8.32	+21.8
4	7.56	8.71	+15.2
40	7.16	8.71	+21.6
480	6.58	6.57	-0.2

Table 3. OLS regression estimation of factors affecting the chlorophyll "a" content of T. erici

4 Days after irradiation

Dependent variable: Chl. 'a' (n=56) R^2=0.60 σ=0.8397 ug

Independent variable	B (g/mg dry wt. cells)	β
KNO_3	2.3000	0.5881
NH_4Cl	1.4287	0.3653
l-Arginine	1.3532	0.3460
$NaNO_3$	1.1340	0.2900
NH_4NO_3	0.9834	0.2515
480 kr	-0.5418	-0.2329

Table 3. continued

8 Days after irradiation

Dependent variable: Chl. 'a' (n=56) R^2+0.82 σ=0.2619 ug

Independent variable	B (g/mg dry wt. cells)	β
KNO_3	1.7525	0.6519
$NaNO_3$	1.6169	0.6015
NH_4NO_3	1.2781	0.4158
NH_4Cl	0.9793	0.3643
l-Arginine	0.6547	0.2436
$(NH_4)2SO_4$	0.4875	0.1814

No independent variable is listed unless it is significant at 0.05.

Table 4. OLS regression estimation of factors affecting the chlorophyll 'b' content of T. erici

4 Days after irradiation

Dependent variable: Chl. 'b' (n=56) R^2=0.68 σ=0.1620 ug

Independent variable	B (g/mg dry wt. cells)	β
KNO_3	0.6222	0.6860
$NaNO_3$	0.3147	0.3470
l-Arginine	0.2962	0.3265
NH_4Cl	0.2301	0.2536
Tryptophan	0.1541	0.1699
l-Lysine	-0.1428	-0.1575

8 Days after irradiation

Dependent variable: Chl. 'b' (n=56) R^2=0.47 σ=0.2659 ug

Independent variable	B (g/mg dry wt. cells)	β
KNO_3	0.4054	0.5436
NH_4Cl	0.2744	0.3680
$NaNO_3$	0.2545	0.3413

Table 5. Chlorophyll content of T. erici cultures after irradiation

Radiation dose (kr)	Chlorophyll 'a' (g/mg dry wt. cells) Days after irradiation 4	8	Percent change
Control	1.467	0.769	-47.6
4	1.305	0.758	-41.9
40	1.290	0.764	-40.8
480	0.812	0.670	-17.5
	Chlorophyll 'b'		
Control	0.352	0.049	-86.1
4	0.228	0.065	-71.5
40	0.249	0.130	-47.8
480	0.217	0.069	-68.2

Values based on means of 3 trials across 14 types of media.

Table 6. Percent reduction in optical density of chlorophyll solutions after acidification with select nitrogen media

Radiation dose (kr)	Reduction in optical density (%) Control	4	40	480	Mean
l-Alanine	40 (0)	40 (0)	40 (0)	40 (0)	40 (0)
l-Arginine	34 (0)	40 (4)	40 (4)	40 (0)	39 (2)
l-Asparagine	40 (0)	34 (7)	40 (0)	40 (0)	38 (2)
Casein	40 (20)	23 (-)	40 (-)	40 (-)	36 (5)
KNO_3	37 (32)	40 (37)	36 (35)	40 (28)	38 (33)
l-Lysine	0 (0)	40 (-)	0 (-)	- (-)	13 (0)
$NaNO_3$	22 (21)	40 (22)	40 (0)	23 (35)	31 (20)
NH_4Cl	40 (7)	31 (19)	18 (0)	34 (0)	31 (7)
NH_4NO_3	24 (0)	30 (0)	14 (16)	13 (0)	23 (4)
$(NH_4)_2SC_4$	23 (0)	40 (0)	40 (7)	0 (0)	24 (2)
No Nitrogen	- (-)	- (-)	- (-)	- (-)	
Peptone	32 (0)	40 (0)	40 (0)	40 (-)	38 (0)
Tryptophan	40 (-)	36 (-)	12 (0)	40 (-)	32 (0)
Urea	40 (18)	0 (0)	40 (36)	40 (0)	30 (14)
Mean within radiation dose	32 (8)	34 (9)	31 (9)	32 (7)	

Unbracketed values - 4 days after irradiation
Bracketed values - 8 days after irradiation
(-) - No pigment present